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CD28 ligation increases macrophage suppression of T cell proliferation

Daniel Silberman¹, Amanda Bucknum¹, Thomas Bartlett¹, Gabriella Composto¹, Megan Kozlowski¹, Amanda Walker¹, Amy Werda¹, Jackelyn Cua¹, Arlene H. Sharpe², John E. Somerville³, and James E. Riggs¹

¹Department of Biology, Rider University, Lawrenceville, NJ 08648

²Department of Pathology, Harvard Medical School, Cambridge, MA 02115

³Immunosciences, Bristol-Myers Squibb, Princeton, NJ 08543

Abstract

When compared to spleen or lymph node cells, resident peritoneal cavity cells respond poorly to T cell activation *in vitro*. The greater proportional representation of macrophages in this cell source has been shown to actively suppress the T cell response. Peritoneal macrophages exhibit an immature phenotype (MHC Class II^{lo}, B7^{lo}) that reduces their efficacy as antigen presenting cells. Furthermore, these cells readily express inducible nitric oxide synthase (iNOS), an enzyme that promotes T cell tolerance by catabolism of the limiting amino acid arginine. Here, we investigate the ability of exogenous T cell costimulation to recover the peritoneal T cell response. We show that CD28 ligation failed to recover the peritoneal T cell response and actually suppressed responses that had been recovered by inhibiting iNOS. As indicated by cytokine ELISpot and neutralizing mAb treatment, this “co-suppression” response was due to CD28 ligation increasing the number of IFN γ -secreting cells. Our results illustrate that cellular composition and cytokine milieu influence T cell costimulation biology.

Keywords

CD28; Costimulation; Macrophages; Suppression

INTRODUCTION

Collaboration between antigen presenting cells (APC) and T lymphocytes is a key checkpoint in the regulation of adaptive immunity. T cell activation requires that APCs provide two signals; processed (peptide) antigen complexed with the Class II major histocompatibility complex to engage the TCR (signal 1) and a costimulatory signal via CD80/86 (B7) engagement of CD28 on the T cell (signal 2).^{1,2} Since the TCR and CD28 are expressed constitutively by resting/naïve T cells appropriate APC expression of Class II MHC and B7 molecules is a major checkpoint for controlling T cell activation. Improper expression of these receptor ligand combinations can promote T cell anergy or apoptosis.³ The great majority of costimulation studies are conducted *in vitro* with low APC:T cell

Correspondence: Dr. J. Riggs, Department of Biology, Rider University, 2083 Lawrenceville Road, Lawrenceville, NJ 08648-3099, USA. riggs@rider.edu.

Current addresses: DS, University of Colorado, Denver, CO; AB, Temple University School of Medicine, Philadelphia, PA; TB, Princeton University, Princeton, NJ; GC, Rutgers University, New Brunswick, NJ; MK, Philadelphia College of Osteopathic Medicine, Philadelphia, PA; A. Walker, University of Medicine and Dentistry of New Jersey; New Brunswick, NJ; A. Werda, Venenum Biodesign, LLC, Hamilton, NJ.

ratios inherent to the natural composition of conventional lymphoid tissue.⁴ There has been little investigation of the effect high APC:T cell ratios could have on T cell activation. This is important to consider because of the paralyzed T cell function seen in tumor microenvironments enriched with immunosuppressive, myeloid cells.⁵

High myeloid: T cell ratios temper T cell function, both at the end of normal immune responses and in tumors where essential T cell effector functions have been abrogated.^{6–11} APCs dampen T cell function by several means, including the expression of enzymes that consume critical amino acids, production of immunoregulatory hormones and cytokines, and generation of regulatory T cells.^{7,9,12} We have shown that cultures of peritoneal cavity (PerC) cells inherently have high macrophage (M ϕ) to T cell ratios (M ϕ :T).^{4,13} IFN γ released by activated T cells triggers the M ϕ s to express indoleamine 2,3-dioxygenase (IDO) and inducible nitric oxide synthase (iNOS), enzymes that inhibit T cell activation by depleting tryptophan and arginine.^{12–14} With their naturally high M ϕ :T cell ratios, these cultures mimic an essential feature of tumor microenvironments. This provides a model to assess immunomodulatory strategies for promoting immunity under conditions of myeloid suppression.

In the studies described herein, we determined whether costimulation could increase the PerC T cell response liberated by inhibiting iNOS. Since PerC M ϕ s have an immature phenotype (MHC Class II^{lo}, B7^{lo}), we reasoned that CD28 ligation would costimulate T cells in these cultures.^{13,15} In contrast, we found that CD28 ligation suppressed the T cell proliferative response. This observation is discussed with respect to the consideration of myeloid-lymphoid ratios in experimental design when assessing the efficacy of immunomodulatory drugs.

MATERIALS AND METHODS

Mice

Two- to four-month old male and female mice, bred and maintained at Rider University, were handled in accord with NIH, Animal Welfare Act, and Rider University IACUC guidelines. Breeding pairs of BALB/c, C57BL/6J, IFN γ RKO (B6.129S7^{Ifng^r}/J), IL10KO (B6.129P2-IL10^{tm1Cyn}/J), iNOSKO (B6.129P2-Nos2^{tm1Lau}/J), CD28KO (B6.129S2-Cd28^{tm1Mak}/J), CD40KO (B6.129P2-Cd40^{tm1Kik}/J), and CD80/86KO or B7KO (B6.129S4-Cd80^{tm1Shr}Cd86^{tm2Shr}/J) mice were obtained from the Jackson Laboratory, Bar Harbor, ME. PDL1KO mice were provided by the laboratory of Dr. Arlene Sharpe, Harvard Medical School, Cambridge, MA.

Preparation of cell suspensions and cell culture

Lymph node (LN) cell suspensions were obtained by gentle disruption of the organ between the frosted ends of sterile glass slides. Peritoneal cavity (PerC) cells were obtained by flushing the peritoneum with 10 mls of warm (37°C) Hanks Balanced Salt Solution supplemented with 3% FCS (Hyclone, Logan, UT). Viable cell counts were determined by Trypan blue exclusion. Various dilutions ($0.33 - 4.0 \times 10^6$ /ml) of cells, in RPMI 1640 culture media (Life Technologies, Grand Island, NY) supplemented with 10% FCS, 0.1 mM nonessential amino acids, 100 U/ml penicillin, 100 μ g/ml streptomycin, 50 μ g/ml gentamicin, 2 mM L-glutamine, 2×10^{-5} M 2-ME, and 10mM HEPES, were incubated in a humidified atmosphere of 5% CO₂ at 37°C in 96-well “U”-, “V”- or flat-bottom microtiter plates (Corning Costar, Fisher Scientific). For anti-CD3 stimulation soluble anti-CD3e mAb (clone 145-2C11)¹⁶ (eBioscience, San Diego, CA), was added at 1.0 μ g/ml. Where exogenous costimulation was tested anti-CD28 (clone 37.51)¹⁷, or an isotype-matched hamster IgG control (eBioscience) was added at 1.0 μ g/ml or B7.1-Fc or B7.2-Fc (R&D

Systems, Minneapolis, MN) were added at 5.0–10.0 µg/ml. Mitogen (ConA) and superantigen (*Staphylococcal* enterotoxin B, SEB) (Sigma Chemical, St. Louis,) were added at 2 and 5 µg/ml, respectively. Anti-IFN γ mAb (XMG1.2, eBioscience) or anti-IL-10 mAb (JES5-2A5, eBioscience) at 5–10 µg/ml were added at culture initiation. Based on prior studies, to inhibit arginine catabolism in IFN γ RKO mice the arginase (ARG) inhibitor *N*- ω -hydroxy-nor-L-arginine (1-NA; CalBiochem, San Diego, CA) was added; to inhibit arginine catabolism in C57BL/6J mice the inducible nitric oxide synthase (iNOS) inhibitor *N*^G-monomethyl-L-arginine (1-MA; CalBiochem) was added.^{12,13} Optimal concentrations of all reagents were determined in titration experiments. Proliferative responses were measured by adding 1 µCi of [³H] thymidine (Moravek Radiochemicals, Brea, CA) after 44 hrs of incubation. The plates were frozen 4 hrs after radiolabeling, then thawed for harvesting onto filter paper mats using a semi-automated cell harvester (Skatron Instruments, Richmond, VA). Radioactivity was measured by liquid scintillation spectrometry. For each experiment 3 – 5 wells were established for each test group.

IFN γ ELISpot

Following overnight incubation of cells plated as described above, IFN γ ELISpot assays were conducted as described by the manufacturer (eBioscience).

Statistical analysis and costimulation index

T cell proliferative responses or number of IFN γ -secreting cells are presented as the average CPM or cell number \pm SEM. All data sets were compared using the Student's *t*-Test with *p* values below 0.05 defined as significant. The costimulation (or co-suppression when values < 1.0) index is defined as the average costimulated (CD3 + CD28 stimulation) CPM divided by the average control (CD3 stimulation alone) CPM. All results are representative of at least three or more independent experiments that generated statistically valid results each time they were conducted.

RESULTS

CD28 ligation costimulates lymph node but not peritoneal cavity T cells

Prior research has shown that resident macrophages (M ϕ s) suppress the activation of PerC T cells.^{4,13} M ϕ -mediated suppression in C57BL/6J PerC cell culture is blocked by the addition of the inducible nitric oxide synthase (iNOS) inhibitor *N*^G-monomethyl-L-arginine (1-MA). Considering that resident PerC M ϕ s are CD80^{lo}, CD86^{lo} we reasoned that T cell costimulation was limiting and that CD28 ligation could enhance the T cell activation evidenced in PerC cell cultures treated with 1-MA.¹³ However the opposite result was observed. While lymph node (LN) cell suspensions responded with an increase in T cell proliferation to increasing concentrations of anti-CD28 (*p* < .005; Fig. 1A), PerC cells exhibited a progressively diminished response (*p* < .05) relative to the control (1-MA alone, no costimulation; Fig. 1B). Although a modest costimulatory response resulted from reducing the number of PerC cells cultured this did not approach that seen with LN cells (Fig. 1C versus 1D; Table 1). Co-suppression was most evident in PerC cultures that increased M ϕ -T cell interaction ("U"-bottom > "V"-bottom > flat-bottom microtiter wells; Table 1). These results illustrate that cell culture composition and density can impact interpretation of T cell costimulation biology.

CD28 ligation co-suppresses superantigen and CD3-independent T cell activation

The high frequency of T cells responsive to CD3 ligation invited speculation as to whether a milder form of T cell stimulation would also be susceptible to CD28-mediated co-suppression. This was the case with the superantigen *Staphylococcus* enterotoxin B (SEB),

which triggered T cell proliferation in the presence of 1-MA and was co-suppressed by CD28 ligation (Fig. 1E). T cell activation independent of CD3 engagement was tested using the mitogenic plant lectin concanavalin A (ConA). This response was also co-suppressed (Fig. 1E) indicating that regardless of how T cells were activated, ligation of the CD28 receptor can, under myeloid-enriched conditions, restrain T cell proliferation.

Co-suppression is not due to Fc binding by the anti-CD28 mAb

To address the possibility that the anti-CD28 mAb triggered suppression via Fc-binding, a species- and isotype-matched, nonspecific mAb was tested. Unlike the hamster anti-CD28 mAb 37.51, the hamster nonspecific control mAb failed to co-suppress T cell proliferation (Fig. 2A). PerC T cells from mice lacking the CD28 receptor (CD28KO) were not affected by addition of the CD28 mAb and the addition of 1-MA did not increase their proliferation (Fig. 2A). Furthermore, the CD28-binding fusion proteins B7.1-Fc and B7.2-Fc both co-suppressed the T cell proliferative response of C57BL/6J PerC cells (Fig. 2B). These observations reinforced that the CD28 receptor can serve as a negative regulator of T cell proliferation.

CD28 ligation co-suppresses BALB/c PerC T cells

Prior research has shown that BALB/c PerC T cells are less suppressed by resident M ϕ than those of C57BL/6J mice.⁴ Consistent with this observation, CD28 ligation co-suppressed BALB/c PerC T cells less than C57BL/6J PerC T cells (CI = 0.65 versus 0.14; Fig. 3). Regardless of the degree of co-suppression, this result illustrated that PerC T cells from two widely studied strains of mice exhibit reduced proliferative responses following CD28 ligation.^{18,19}

Role of IFN γ in CD28 co-suppression

Since costimulation is known to increase T cell cytokine production we assessed whether increased production of a regulatory cytokine could be the mechanism for CD28-mediated co-suppression.²⁰ IL-10 and IFN γ are hallmark regulatory cytokines so the role of these molecules was tested. The CD28-mediated co-suppression of PerC T cells from IL-10KO mice was no different than that seen for wild type (C57BL/6J) mice (Fig. 4A). In contrast, PerC T cells from IFN γ RKO mice were less suppressed by M ϕ s and were costimulated by CD28 ligation (Fig. 4B). Direct evidence in C57BL/6J mice that IFN γ plays a role in co-suppression was provided by the observation that the addition of a neutralizing anti-IFN γ mAb released PerC T cells from M ϕ -suppression and negated CD28-mediated co-suppression. Neutralizing mAbs for IL-10 and IL-4 had no effect on recovering the T cell proliferative response (Fig. 4C).

CD28 ligation increases the number of IFN γ -secreting cells

IFN γ ELISpot assays were employed to measure the impact of CD28 ligation on IFN γ -secreting cell (IFN γ SC) number. CD28 ligation costimulated an increase in the number of IFN γ SCs, particularly at low cell density, and the addition of 1-MA had little effect, particularly at increased cell density (Fig. 5A). Consistent with the observation of less co-suppression of BALB/c PerC T cell proliferation (Fig. 3), there were fewer IFN γ SC in this strain (Fig. 5B). Although BALB/c PerC T cells consistently exhibited a greater costimulatory response, C57BL/6J PerC cells always had the greater number of IFN γ SC (Fig. 5B). C57BL/6J PerC cells exhibited the greatest numbers of IFN γ SC when a neutralizing anti-IL10 mAb was included during their generation, particularly at high cell density (Fig 5C). However, the greatest costimulatory increase in IFN γ SC number followed CD28 ligation of LN cells (CI = 7.75, Fig. 6A). This increase occurred without suppression of LN T cell proliferation (Fig. 1). Likewise, IFN γ RKO PerC T cells, which had greater

numbers of IFN γ SC than C57BL/6J mice, were costimulated for both proliferation (Fig. 4B) and IFN γ SC number (Fig. 6B). These results illustrate that while CD28 ligation increases IFN γ SC production, both the cellular composition and the cytokine milieu of the culture determine whether the T cell proliferative response will be costimulated or co-suppressed.

Role of cell surface molecules in CD28 receptor-mediated co-suppression

IFN γ can increase the expression of molecules that either promote (CD40, CD80/B7.1, CD86/B7.2) or inhibit (CD274/B7H1/PDL1) T cell activation.²¹ To determine if these molecules have a role in the T cell biology described in the preceding experiments, the PerC cells of CD40KO, B7KO, and PDL1KO mice were studied. While CD40KO and PDL1KO mice exhibited co-suppression analogous to that of C57BL/6J mice (Fig. 7), the PerC T cells of B7KO mice were more similar to those of CD28KO mice (Fig. 2A) in that the addition of 1-MA did not increase the T cell proliferative response (Fig. 7B). Furthermore, B7KO PerC T cell proliferation was costimulated by CD28 ligation. Both CD28KO and B7KO mice had fewer IFN γ SC than wild type C57BL/6J mice (Fig. 8). Although costimulation significantly increased IFN γ SC number for the B7KO, the small number of these cells did not temper the proliferative response (Fig. 7B). These data reinforce that the CD28-B7 receptor-ligand pathway can trigger immune suppression via increased production of IFN γ .

CD28 receptor-mediated co-suppression requires iNOS

IFN γ has been shown to inhibit T cell proliferation by triggering M ϕ s to increase expression of the arginine-consuming enzyme iNOS.^{11,13} To assess the role of iNOS in CD28-mediated co-suppression, PerC T cells from iNOSKO mice were studied. The data show that iNOS is essential for M ϕ -mediated T cell suppression and that CD28-triggered co-suppression does not occur for this strain (Fig. 9A). As a direct test of the role of iNOS in C57BL/6J mice, their PerC cells were titrated and tested for co-suppression with graded concentrations of the iNOS inhibitor 1-MA. The data show co-suppression at the highest (1.0×10^5 /well; CI = .30) and costimulation at the lower (0.33 and 0.11×10^5 /well) concentrations of PerC cells tested (Fig. 9B). However, co-suppression at the intermediate cell concentration (0.33×10^5 /well) returned as the inhibitor was diluted (CI values, relative to the 1 mM 1-MA control: CI_{1.0} = 1.34, CI_{0.5} = .91, CI_{0.25} = .72, CI_{0.125} = .28). There was only costimulation at the lowest PerC cell concentration (CI = 2.03). These data reinforce that iNOS is the mechanism for co-suppression and that cognate myeloid-lymphoid interaction is an essential element of this form of T cell regulation.

DISCUSSION

The failure of PerC T cells to proliferate in response to CD3 ligation is not an intrinsic T cell defect nor due to APC immaturity as T cell purification and PerC cell titration can rescue this response (Fig. 1D).^{4,13} A surplus of natural costimulation, due to increased formation of immunological synapses inherent to the APC-rich composition of PerC cells, triggered a natural “braking system” with IFN γ production promoting iNOS expression, arginine catabolism, and lymphocyte proliferative paralysis.^{11,14} CD28 ligation, rather than reversing this pathway, supplemented the natural costimulatory response and increased IFN γ production and immune paralysis. This “co-suppression” revealed the significance of the myeloid:lymphoid composition of the cellular preparation targeted for costimulation. This observation is particularly relevant to current efforts to deploy immunomodulatory drugs to amend the aberrant immune regulation that is a hallmark of myeloid-rich tumor microenvironments.²² *In vitro* screening assays that can reproduce the immunosuppressive elements of tumor microenvironments will be essential to facilitate effective drug development.^{5,10,23–28}

The same anti-CD28 mAb (clone 37.51, *ref.* 17) that all prior *in vitro* research has revealed as costimulatory has been shown to inhibit T cell expansion and cytokine production *in vivo*.^{29–31} Another anti-CD28 mAb (clone JJ319, *ref.* 32) tempers acute GVHD.^{33–35} In these studies, CD28 blockade was thought to promote allograft tolerance by negating CD28/B7 interaction or by allowing CTLA-4/B7 interaction to costimulate IFN γ production and IDO/iNOS expression.^{31,34,35} Since CTLA-4 ligation has been shown to restrict IFN γ production the tolerance observed more likely reflects a co-suppressive response, a hypothesis validated by research showing that *in vivo* administration of the 37.51 mAb activated T cells to produce the IFN γ essential for tolerance.^{31,36} Likewise, increased numbers of IFN γ SC and regulatory T cells (T_{regs}) were noted following *in vivo* administration of the anti-CD28 mAb E18 and a monovalent Ab (Sc28AT) promoted allograft tolerance by increasing IDO and T_{regs}.^{37,38} PerC T_{regs} are not a factor with *in vitro* co-suppression because PerC cells from T cell-deficient *nude* and *scid* mice suppress exogenous T cell proliferation via iNOS.¹³ The generation of Foxp3⁺ T cells is unlikely in short-term culture, particularly with IFN γ and nitric oxide suppressing their generation.^{14,39–42} There is evidence however, that IFN γ -generated regulatory APCs can promote T_{reg} development, a factor more likely in longer term, *in vivo* models of T cell tolerance.^{43,44}

In vivo administration of 37.51 to BALB/c mice afforded protection from lethal septic shock via IL-10-mediated inhibition of TNF α production.⁴⁵ This observation is consistent with the T cell cytokine biology of BALB/c (Th2/IL10) versus (Th1/IFN γ) C57BL/6J mice. The lower number of IFN γ SC and less co-suppression witnessed with BALB/c PerC cells (Figs. 3, 5B) could be due to cytokine antagonism via autocrine IL-10 production by PerC B-1 B cells or B_{regs}.^{23,46} In support of this premise, B-1 B cell-deficient BALB.*xid* mice have PerC IFN γ SC numbers more similar to C57BL/6J mice rather than to BALB/c mice and exhibit co-suppression responses most like C57BL/6J mice.⁴ IL-10 still restrained PerC IFN γ SC production in C57BL/6J mice, particularly at higher cell density (Fig. 5C). These results reinforce that culture density and cellular composition are important factors when interpreting T cell suppression biology. Although the resolution of T cells into Th1/Th2/Th17/T_{reg} subsets is well established, the functional plasticity of M ϕ s confounds their simple categorization as classically (M1) or alternatively activated (M2) cells.^{11,47,48} There is growing appreciation for the heterogeneity of myeloid cells being a key factor in the generation of distinct T cell subsets.⁴⁹

Direct evidence that the CD28/B7 pathway can temper immunity came with the observation that CD28KO and B7KO PerC T cells were not suppressed at culture densities that tempered C57BL/6J and PDL1KO T cell proliferation (Figs. 2, 7). Peripheral T cell viability depends upon the CD28/B7 pathway as both CD28KO and B7KO mice had reduced numbers of PerC CD4⁺ and CD8⁺ T cells relative to C57BL/6J controls.⁴ PerC IFN γ SC numbers were low for both of these mutants (Fig. 8) and 1-MA was not required to inhibit iNOS and reveal their proliferative response (Fig. 7). Even with costimulation, B7KO PerC IFN γ SC numbers did not reach the levels seen with BALB/c mice, which were sufficient to temper T cell proliferation. Although the greatest number of IFN γ SC were found in IFN γ RKO mice their PerC T cells responded to anti-CD3 and were costimulated by CD28 ligation (Fig. 6B) revealing the critical role of IFN γ signaling for suppression. Although these results suggest that T cells can be expanded in M ϕ -rich environments, these cells may not be the IFN γ -dependent effectors required for an optimal anti-tumor response.^{41,50,51}

That PDL1KO PerC T cells were suppressed was surprising considering that PDL1 expression has been shown to restrict T cell activation in lymphoid and normal tissue as well as in tumors.^{52–54} Since PDL1 costimulates IL-10 production the absence of this ligand likely enhanced IFN γ production and thus co-suppression (Fig. 5C).⁵⁵ In a similar fashion,

fibroblastic reticular cells from PDL1KO mice were recently shown to have increased IFN γ -dependent, iNOS-mediated T cell suppression relative to wild type control cells.⁵⁶ Although the CI was lower for PDL1KO PerC T cells relative to the C57BL/6J control (Fig. 7B) the proliferative differences were not statistically significant between these groups. Prior research has shown that neutralization of PDL1 on BALB/c M ϕ s leads to T cell proliferative arrest by increasing IFN γ /iNOS production, however CD28 costimulation was not assessed in this study.⁵⁷ Although these results suggest that PDL1 is not a significant factor in IFN γ -M ϕ -mediated suppression, this molecule and other B7 homologs are clearly important in other regulatory pathways.^{2,54,58–61} The complexity and variety of T cell-APC and T cell-T cell interactions among the B7 family members insure that there is more to learn regarding this important family of costimulatory/co-inhibitory molecules.^{2,59–61}

In summary, depending upon the myeloid composition of the target tissue, CD28 ligation can suppress rather than costimulate T cell proliferation. Although this pathway may be more potent than CTLA-4-Ig in controlling T cell activation, the “cytokine storm” that ensued following *in vivo* trials of a superagonist anti-CD28 mAb might have tempered enthusiasm for this strategy.^{31,62,63} This case certainly has made it clear that additional models must be developed to assess the safety of immunomodulatory biopharmaceuticals.^{64–67}

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Abbreviations used in this article

ARG	arginase
CI_{PerC}	costimulation index for peritoneal cavity T cells
IFNγSC	IFN γ -secreting cells
iNOS	inducible nitric oxide synthase
KO	knockout
LN	lymph node
PerC	peritoneal cavity
1-MA	<i>N</i> ^G -monomethyl-L-arginine (iNOS inhibitor)
1-NA	<i>N</i> - <i>w</i> -hydroxy-nor-L-arginine (arginase inhibitor)

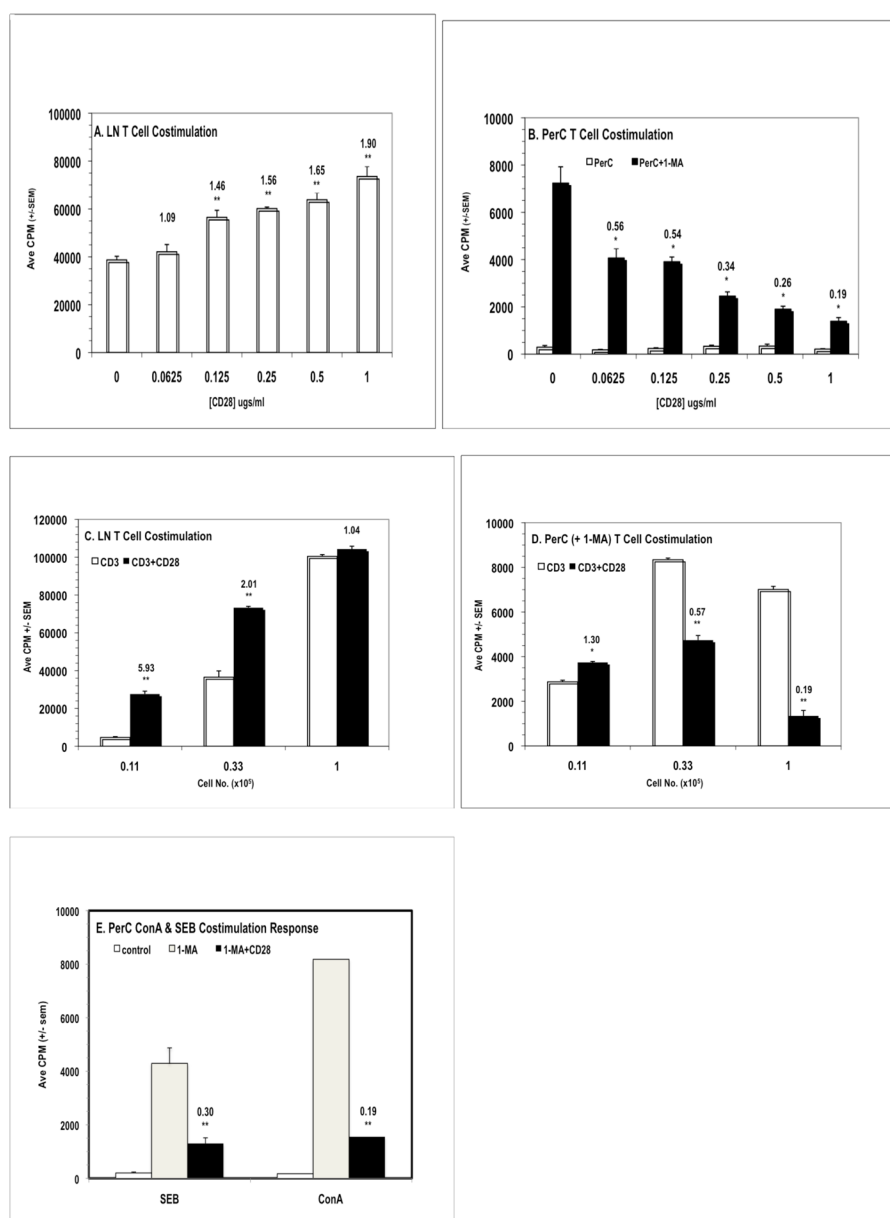
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**Figure 1.**

CD28 ligation costimulates lymph node T cell proliferation but suppresses the peritoneal cavity T cell response. Panels A & B: effect of anti-CD28 concentration. 2×10^5 C57BL/6J LN (A) or PerC cells, +/- 1-MA (B), were cultured with anti-CD3 +/- CD28 costimulation. The numbers above the histograms are costimulation indices (CI) which represent the average CPM for the costimulated response divided by the average CPM for the uncostimulated response; for PerC cells CI values represent ratios of the costimulated PerC + 1-MA response to the control (uncostimulated) PerC + 1-MA response. * = $p < .05$; ** $p < .005$. The addition of 1-MA had no effect on LN T cell proliferation; anti-CD28 in the absence of anti-CD3 had no effect on LN or PerC T cell responses. Panels C & D: effect of cell concentration on the CD28 costimulation response. Titered numbers of C57BL/6J LN or PerC cells + 1-MA were cultured +/- CD28 costimulation. CI values reflect comparisons within each cell concentration. Panel E: CD28 ligation co-suppresses the PerC T cell

response to SEB and ConA. 1.25×10^5 PerC cells, +/- 1-MA, were cultured with SEB or ConA. CI values represent the ratios of the costimulated PerC + 1-MA response to the control (uncostimulated) PerC + 1-MA response. LN responses to SEB were $17,597 \pm 405$ (- CD28) & $19,916 \pm 409$ (+ CD28) and to ConA were $42,977 \pm 4314$ (- CD28) & $38,604 \pm 3395$ (+ CD28). Each experiment is representative of at least three independent experiments that yielded comparable, statistically valid results.

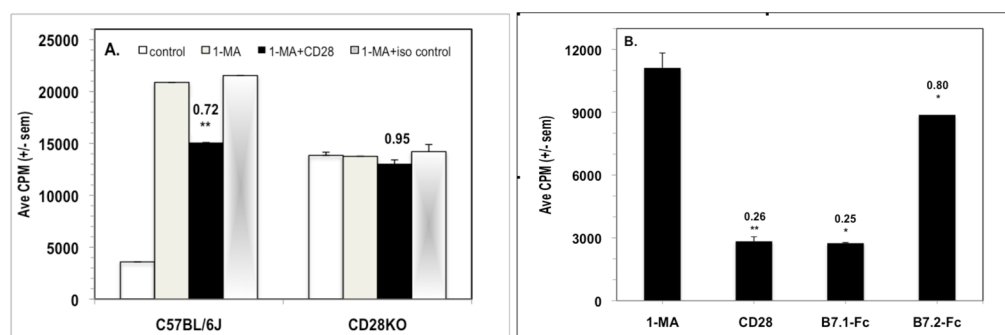


Figure 2.

CD28 triggered co-suppression is not due to Fc binding by the anti-CD28 mAb. For Panel A, 1.25×10^5 C57BL/6J or CD28KO PerC cells +/- 1-MA were cultured with anti-CD3 +/- anti-CD28 mAb costimulation or with an isotype- and species-matched, nonspecific mAb. CI values represent the ratio of the costimulated PerC + 1-MA response to the control (uncostimulated) PerC + 1-MA response. * = $p < .05$; ** $p < .005$. For Panel B, 1.0×10^5 C57BL/6J PerC cells were cultured with anti-CD3 + 1-MA alone or with CD28, B7.1Fc, or B7.2Fc costimulation. CI values represent the ratio of the costimulated response to the control (1-MA alone) response. Each experiment is representative of four independent trials that produced similar results.

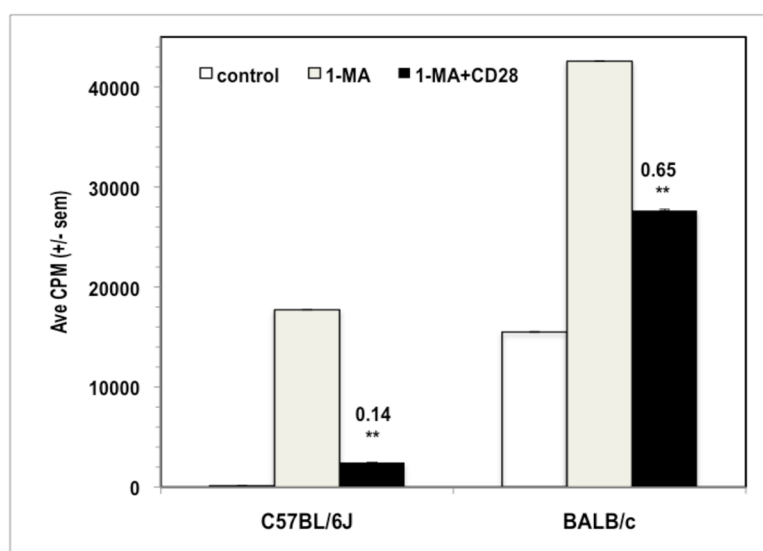
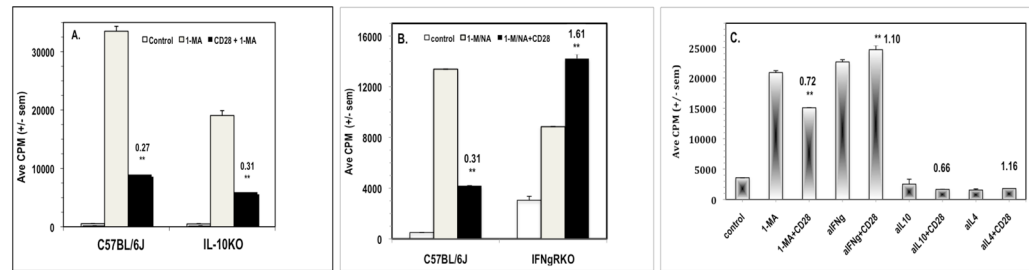
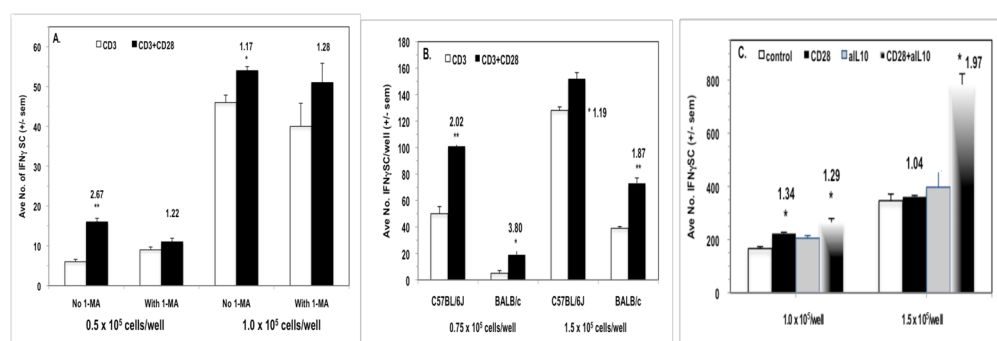


Figure 3.

CD28 ligation suppresses the BALB/c peritoneal cavity T cell proliferative response. 1.5×10^5 C57BL/6J or BALB/c PerC cells +/- 1-MA were cultured with anti-CD3 +/- anti-CD28. CI values represent the ratio of the CD28 costimulated response to the 1-MA alone response. * = $p < .05$; ** $p < .005$. The data presented are representative of six independent experiments that yielded similar results.

**Figure 4.**

Role of IL-10 and IFN γ in CD28 triggered co-suppression. For Panel A, 1.5×10^5 C57BL/6J or IL10KO PerC cells +/- 1-MA were cultured with anti-CD3 +/- anti-CD28. For Panel B, 1.5×10^5 C57BL/6J PerC cells +/- 1-MA or IFN γ RKO PerC cells +/- the arginase inhibitor 1-NA were stimulated with anti-CD3 +/- anti-CD28. CI values represent the ratio of the CD28 costimulated response to the 1-MA or 1-NA (IFN γ RKO) alone response. The data presented are representative of three experiments with IL10KO mice and seven with IFN γ RKO mice. For Panel C, 1.25×10^5 C57BL/6J PerC cells alone or with 1-MA or with neutralizing mAbs for either IFN γ , IL-4, or IL-10, were cultured with anti-CD3 +/- anti-CD28 costimulation. The CI values represent the ratios of the CD28 costimulated response to the 1-MA alone or the anti-cytokine mAb alone response. ** $p < .005$. The data presented are representative of five independent trials that yielded similar results.

**Figure 5.**

CD28 ligation increases the number of peritoneal cavity IFN γ -secreting cells. For Panel A, 0.5 and 1.0×10^5 C57BL/6J PerC cells \pm 1-MA were cultured with anti-CD3 \pm anti-CD28 as described in Methods. For Panel B, 0.75 and 1.5×10^5 C57BL/6J or BALB/c PerC cells were cultured with anti-CD3 \pm anti-CD28. For Panel C, 1.0 and 1.5×10^5 C57BL/6J PerC cells alone or with a neutralizing anti-IL-10 mAb were cultured with anti-CD3 \pm anti-CD28 costimulation. All CI values represent the ratio of the costimulated response to the uncostimulated response. * = $p < .05$; ** $p < .005$. The data shown are representative of three separate experiments that generated similar results.

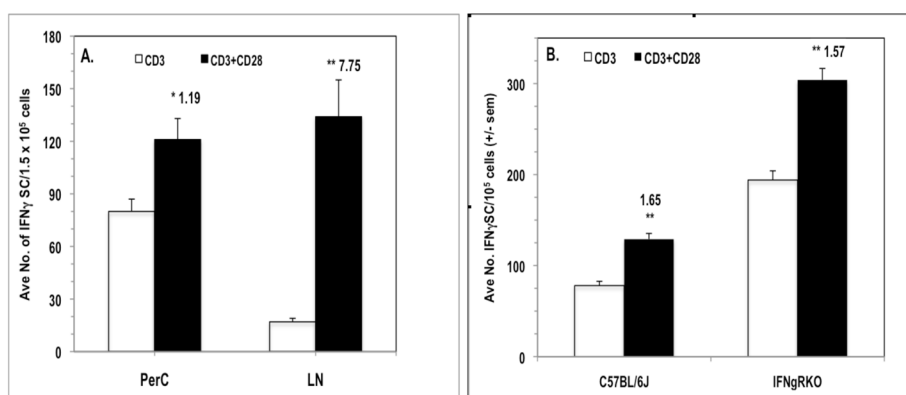


Figure 6.

CD28 ligation increases IFN γ -secreting cell number for cells that exhibit a proliferative costimulatory response. For Panel A, 1.5×10^5 C57BL/6J PerC or LN cells were stimulated with anti-CD3 +/- anti-CD28 costimulation. In Panel B, 1.0×10^5 C57BL/6J or IFN γ RKO PerC cells were stimulated with anti-CD3 +/- anti-CD28 costimulation. CI values represent the ratio of the costimulated response to the uncostimulated response. * = $p < .05$; ** $p < .005$. The data shown are representative of three separate experiments that generated similar results.

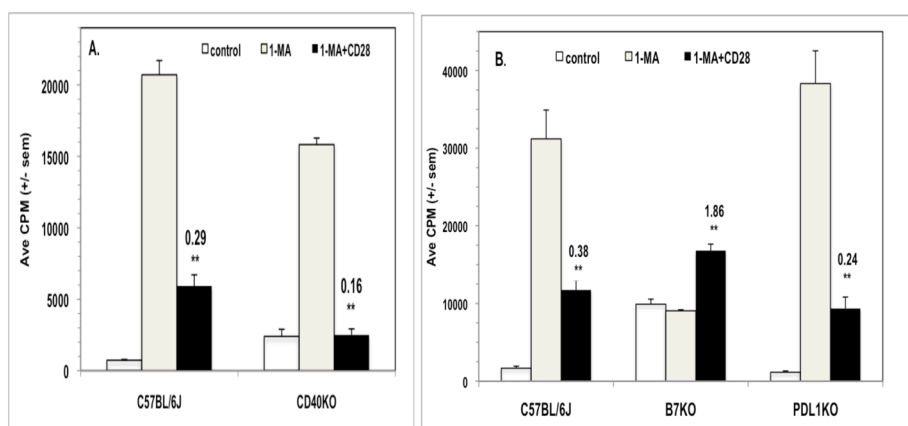


Figure 7.

CD28 ligation co-suppresses peritoneal cavity T cells from CD40KO and PDL1KO mice but costimulates those from CD80/86KO (B7KO) mice. For both Panels, 1.5×10^5 PerC cells +/- 1-MA were stimulated with anti-CD3 +/- anti-CD28 costimulation. CI values represent the ratio of the CD28 costimulated response to the 1-MA treated, uncostimulated response. ** $p < .005$. The data shown are representative of three independent CD40KO experiments & eight separate PDL1KO experiments.

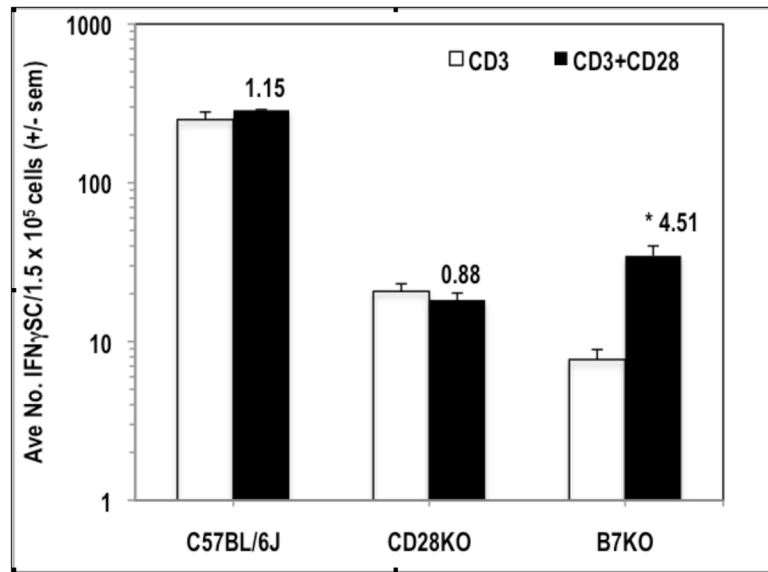


Figure 8.

CD28KO and B7KO mice have low numbers of IFN γ -secreting cells in their peritoneal cavity. 1.5×10^5 PerC cells were stimulated with anti-CD3 +/- anti-CD28. CI values represent the ratio of the CD28 costimulated response to the control response. * $p < .05$. The data shown are representative of three separate trials that yielded similar data.

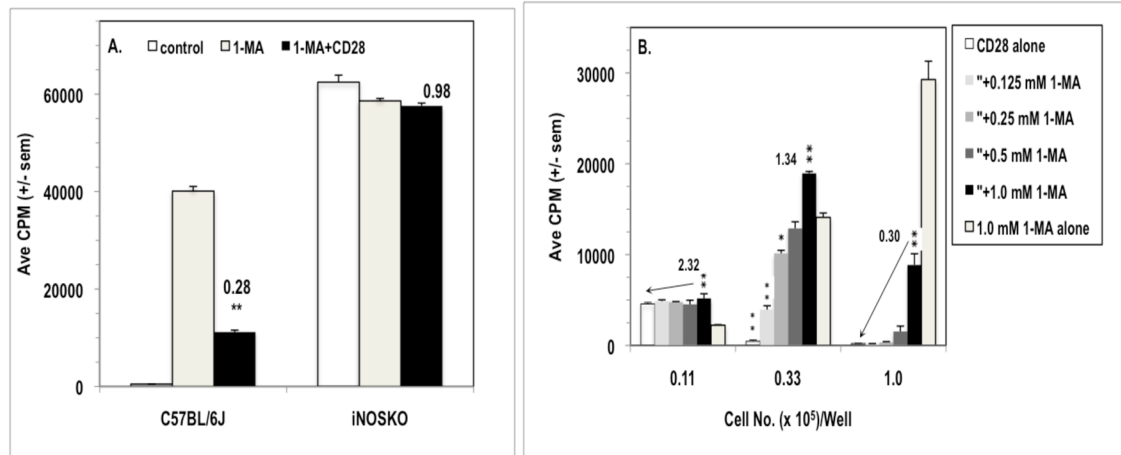


Figure 9.

CD28 cosuppression requires iNOS. In Panel A, 1.5×10^5 C57BL/6J or iNOSKO PerC cells +/- 1-MA were stimulated with anti-CD3 +/- anti-CD28. CI values represent the ratio of the CD28 costimulated response to the 1-MA treated, uncostimulated response. In Panel B, titrated numbers of C57BL/6J PerC cells cultured either alone or with titrated concentrations of 1-MA, were stimulated with anti-CD3 +/- anti-CD28. CI values represent the ratio of the CD28 costimulated response to the 1-MA treated, uncostimulated response at the highest 1-MA concentration (1.0 mM). For both panels * = $p < .05$; ** $p < .005$. The arrows in Panel B indicate that the p values carry down the 1-MA titration series. The data shown in Panel A are representative of five similar experiments, for Panel B for three similar experiments.

Table 1Costimulation Indices for Lymph Node and Peritoneal Cavity T Cells¹

Lymph Node Cells²	<u>0.66 × 10⁵/well</u>	<u>0.22 × 10⁵/well</u>	<u>0.07 × 10⁵/well</u>
<i>Flat-Bottom</i>	1.35	5.44	14.28
<i>U- Bottom</i>	1.31	1.61	3.14
<i>V-Bottom</i>	1.32	1.61	2.01
Peritoneal Cells³	<u>0.66 × 10⁵/well</u>	<u>0.22 × 10⁵/well</u>	<u>0.07 × 10⁵/well</u>
<i>Flat-Bottom</i>	1.17	<u>2.42</u>	1.81
<i>U- Bottom</i>	<u>0.66</u>	0.83	1.20
<i>V-Bottom</i>	<u>0.73</u>	1.15	<u>1.96</u>

¹C57BL/6J LN or PerC cells were cultured with anti-CD3 + 1-MA (PerC) +/- anti-CD28. CI values determined as described in Methods; *p* values reflect comparisons of +/- costimulation. C57BL/6J LN cells were 2–3% myeloid, 85–90% lymphoid (58% CD3⁺, CD4:CD8 = 1.13); PerC cells were 40–45% myeloid, 25–30% lymphoid (18% CD3⁺, CD4:CD8 = 1.82)⁴

²All LN values *p* < .005

³Underlined PerC values *p* < .05